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Asymptomatic Dystrophinopathy

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A 4-year-old girl was referred for evaluation for a mild but persistent serum aspartate aminotransferase (AST) elevation detected incidentally during routine blood screening for a skin infection. Serum creatine kinase activity was found to be increased. Immunohistochemical study for dystrophin in her muscle biopsy showed results consistent with a carrier state for muscular dystrophy. Molecular work-up showed the proposita to be a carrier of a deletion mutation of exon 48 of the dystrophin gene. Four male relatives also had the deletion mutation, yet showed no clinical symptoms of muscular dystrophy (age range 8–58 yrs). Linkage analysis of the dystrophin gene in the family showed a spontaneous change of an STR45 allele, which could be due to either an intragenic double recombination event, or CA repeat length mutation leading to identical size alleles. To our knowledge, this is the first documentation of an asymptomatic dystrophinopathy in multiple males of advanced age. Based on molecular findings, this family would be given a diagnosis of Becker muscular dystrophy. This diagnosis implies the development of clinical symptoms, even though this family is clearly asymptomatic. This report underscores the caution which must be exercized when giving presymptomatic diagnoses based on molecular studies. Am. J. Med. Genet. 69:261–267, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: Becker muscular dystrophy; dystrophin; hyperCKemia; aspartate aminotransferase (AST); alanine aminotransferase (ALT); double recombination

INTRODUCTION

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities are frequently determined in clinical practice, and elevations are usually considered indicative of hepatic disease or damage. Patients with normal liver function have occasionally been found with elevated AST/ALT. As large amounts of the transaminases (particularly AST) are present in muscle, serum AST elevations can reflect an underlying myopathy [Wroblewski et al., 1959; Schwarz et al., 1984; Odièvre et al., 1987; Morse et al., 1987].

Duchenne muscular dystrophy and the milder Becker muscular dystrophy invariably show high circulating muscle creatine kinase levels, and transaminases also may show elevations above the normal range [Ebashi et al., 1959; Shaw et al., 1967; Munsat et al., 1973]. Transaminases are included in routine blood panels more frequently than creatine kinase, and as a result it is possible to ascertain a presymptomatic Duchenne/Becker muscular dystrophy patient based solely on AST/ALT levels [Schwarz et al., 1984; Odièvre et al., 1987; Morse et al., 1987]. We have seen patients evaluated for liver disease (including liver biopsy) before a primary muscle disorder was detected (EP Hoffman, unpublished observations).

Duchenne and Becker muscular dystrophy (DMD)/(BMD) are X-linked recessive diseases caused by mutations in the gene encoding "dystrophin", a high molecular weight muscle cytoskeletal protein [Hoffman et al., 1987]. DMD is a severe form of the disease in which the protein is completely missing [Hoffman et al., 1988]. BMD is milder and less prevalent clinical variant of DMD: dystrophin is present but altered [Hoffman et al., 1989]. The dystrophin protein (427kDa) is encoded by the largest gene, by far, identified to date, which spans approximately 2.4 Mb of Xp [Koenig et al., 1987]. This large size is at least partly responsible for the gene's high mutation rate (>1 in 10^4 meioses), and its very high frequency of intragenic recombination

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[Oudet et al., 1992]. Two thirds of mutations are gene deletions or duplications, the remaining are presumed to be point mutations [Hoffman, 1993]. It is considered very important to identify the molecular basis of the disease in DMD and BMD patients for correct diagnosis of the proband, and for the screening of other affected males and female carriers in the family.

We report the case of a girl in whom evaluation of a slight transaminase elevation led to the discovery that she was an asymptomatic carrier of a dystrophinopathy. By CK determination and molecular genetic analysis of other relatives we were able to identify 4 males between the age of 8 and 58 affected by an asymptomatic dystrophinopathy, as well as 7 female carriers. We also present evidence for a possible intragenic double recombination event, and the first example of asymptomatic dystrophinopathy in older males. Each of the three affected adult males was shown to have a subclinical cardiomyopathy.

MATERIALS AND METHODS

Case Report

A 4-year-old girl was found to have a mild elevation of AST (81 U/l; normal <40 U/l) and ALT (43 U/l; normal <36 U/l) or routine laboratory evaluation for a skin infection, and was admitted to Meyer Pediatric Hospital in Florence, Italy for further investigations for a possible hepatic disease. Review of her clinical records showed previous mild increases of transaminase activity when she was 11 months old (AST 76 U/l, ALT 40 U/l), at which time she was experiencing mild recurrent upper respiratory infections.

The patient (Fig. 1, IV-2) and her parents denied anorexia and easy fatiguability. There was no history of medication use or recent intramuscular injections. There was no family history of either liver or muscle disease. On physical examination, the girl was thin, but appeared healthy. Liver size was normal, and there were no cognitive deficits, no clinical signs of hypothyroidism, and no muscle weakness. Mild calf hypertrophy was noted.

Laboratory studies demonstrated: AST 62 U/L, ALT 25 U/L, creatine kinase (CK) 493 U/L (normal < 160), and lactate dehydrogenase (LDH) 686 U/L (normal < 450). Hepatitis serologies were negative. Repeat measurements confirmed the AST and CK elevations. An electromyography (EMG) study was within normal limits. A muscle biopsy was performed. Light and electron microscopic findings were consistent with a mild myopathic process, with some fiber size variation and central nuclei.

Aminotransferase and CK levels in other relatives were determined (Fig. 1). Four male relatives, related

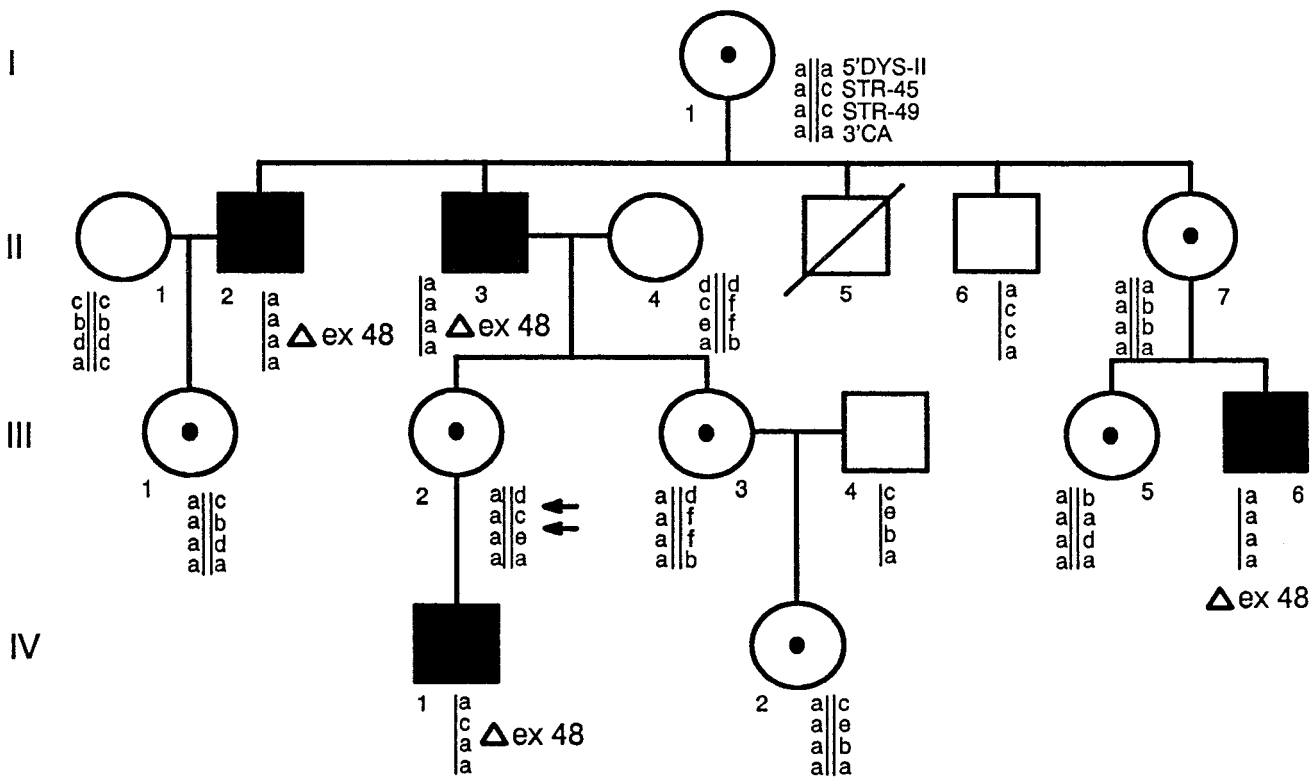

Fig. 1. Pedigree of the family studied, with molecular DNA deletion and linkage results. Shown is the pedigree of the family of the proposita (IV-2) detected through incidental finding of elevated AST levels. Molecular analysis showed her to be a carrier of an exon 48 deletion (Δ ex48) of the dystrophin gene. Males carrying the deletion are shown as affected, however all males were asymptomatic when studied. Female carriers of the deletion are also shown. Shown are dystrophin gene linkage studies using 4 CA repeat polymorphisms (5′DYS-II, STR-45, STR-49, 3′CA). Haplotypes are shown in phase, with the "aaaa" haplotype representing the at-risk dystrophin gene. The two arrows in female carrier III-2 indicate the sites of the double recombination event or a new mutation of the "a" STR-45 allele to a "c" allele leading to child IV-1.

TABLE I. Summary of Clinical and Laboratory Findings in the Asymptomatic Dystrophinopathy Family*

Patient	Age	AST	ALT	CK	Clinical Findings	EMG	Cardiology
II-1	51	45	12	802	Myalgia, calf hypertrophy	Normal	Subclinical cardiomyopathy
II-2	58	46	24	325	Calf hypertrophy	N/A	Subclinical cardiomyopathy
III-6	28	31	26	477	Calf hypertrophy	Normal	Subclinical cardiomyopathy
IV-1	8	62	33	2835	Calf hypertrophy	Myopathic	Normal

*AST, aspartate aminotransferase; ALT, serum alanine aminotransferase; CK, creatinine kinase.

to the proposita through maternal lineages showed CK and AST elevations (Table I). With the exception of the proposita, all female relatives had normal CK and transaminase levels.

Clinical, electromyographic, and cardiac evaluations were performed in the 4 males with CK elevations (Table I). No cramps, no easy fatiguability, no winging of the scapulae, and no joint contractures were present; all showed calf hypertrophy with normal muscle tone and strength. Three of the males were adult laborers, and one of them (III-6) was actively involved in amateur athletics. Recently, patient II-1 reports myalgias, but the other males deny experiencing any muscle pain.

Electromyographic (EMG) studies were normal in II-1 and III-6. EMG studies of the IV-1 of the deltoid, tibialis anterior, and quadriceps femoris (vastus lateralis) muscles showed absent resting activity, reduction of the duration and amplitude of the motor units and alteration of recruitment patterns. EMG studies were refused by II-2.

Clinical cardiac findings and ECG were normal in all males. In male II-2 and II-3, echocardiogram showed mild dilated left ventricle (left ventricular end-diastolic dimension 58 and 59 mm respectively; normal < 55mm) and diminished systolic function (fractional shortening 32% and 22% respectively, normal 30–42%; left ventricular ejection fraction 56% and 43% respectively, normal >60%; mean velocity of circumferential fiber shortening 0.9 circumference per second [circ/sec] in both cases, normal >1). Slightly diminished systolic function was observed in III-6 (fractional shortening 32%; left ventricular ejection fraction 53%; mean velocity of circumferential fiber shortening 1.02 cir/sec). Echocardiogram was normal in the young boy (IV-1). A muscle biopsy was performed in male IV-1 at age 8 yrs. The biopsy showed mild variability in fiber size, largely due to the hypercontracted fibers. There was a slight increase in the number of central nuclei and rare degenerating fibers.

Dystrophin Protein Analysis

A biopsy of the vastus lateralis (quadriceps femoris) muscle was performed in the patients (IV-1, IV-2) and a portion was frozen in liquid nitrogen, as part of clinical evaluation for muscular dystrophy. Dystrophin immunofluorescence analysis was done as previously described [Mirabella et al., 1993] using three antidystrophin monoclonal antibodies (Ab) against the N-terminal, mid-rod and C-terminal regions.

Dystrophin immunoblotting was done using, as primary antibodies, monoclonal Ab anti-mid-rod region [Mirabella et al., 1993].

Dystrophin Gene Analysis

DNA was isolated from peripheral blood, collected in EDTA, of relatives, as described previously by Higuchi [1989].

Multiplex PCR analysis was used to identify possible deletions in the males of the family; 18 exons of the dystrophin gene were screened as previously described [Chamberlain et al., 1988; Beggs et al., 1990]. PCR products were separated either on 1% agarose 2% low-melt agarose or 1,4% agarose gels.

The multiplex assays frequently used employ intronic PCR primers flanking specific exons. Additional exonic primers were synthesized for exons 12 and 48 (exon48-F: 5′-tttccagagctttacctga-3′; exon48-R: 5′-actgattcctaataggaga-3′; exon12-F: 5′-acatagagttttaatggatct-3′; exon12-R: ′-gaggctcttcctccattt-3′).

Dosage studies of deleted exon 48 were performed in all females by multiplex fluorescent PCR [Schwartz et al., 1992; Hoop et al., 1994].

Multiplex PCR using fluorescently labeled primers for four intragenic CA repeat polymorphisms [5′CA, Feener et al., 1991; STR-45 and STR-49, Clemens et al., 1991; 3′CA, Oudet et al., 1990] was performed in all relatives and analyzed on an automated sequencer, as described previously [Schwartz et al., 1992, Hoop et al., 1994]. Additional polymorphisms in the dystrophin gene were used to determine if additional loci showed genotypes consistent with a double recomination event. An intragenic CA-repeat polymorphism in intron 44 (STR-44) was analyzed in the proband, his mother, his grandfather and grandmother [Clemens et al., 1991]. In addition, STR-45 PCR products were tested for non-CA repeat polymorphisms doing SSCP analysis [Orita et al., 1989].

PCR amplification was also done for analysis of intragenic pERT-87 RLFPs using the following enzymes: BamHI, TaqI, and XmnI [Roberts et al., 1989].

mRNA Analysis

Total RNA was isolated from 10 mg of flash-frozen muscle biopsy (IV-1) by guanidium thiocyanate homogenization followed by phenol-chloroform extraction.

RNA integrity and concentration were checked by agarose gel electrophoresis. Approximately 500 ng of RNA was reverse transcribed into single stranded cDNA, as described previously [Fidzianska et al., 1995].

Nested RT-PCR was carried out using a set of 10 overlapping PCR primer sets covering the dystrophin RNA, as described previously [Roberts et al., 1991]. A second round of nested RT-PCR was carried out using the first round PCR products as template. Both rounds of RT-PCR products were checked on 1.5% agarose gels. The complete set of 40 primers used for this analysis were as described previously [Roberts et al., 1991].

The nested RT-PCR product containing exon 48 (fragment 7c–7d) was excised from an agarose gel and purified (Quiaquick kit; Quiagen Inc.). Approximately 100 ng of purified PCR product was used as a template for di-deoxy sequencing. The sequence of the double stranded PCR product was performed on both strands using the following primers: 47F (6,970–6,990bp of dystrophin cDNA), and 49R (7,386–7,404bp). The sequencing reactions were done using AmplyCycle kits (Perkin Elmer), with incorporation of radiolabeled (α^{32}P)dATP. Products from the sequencing reactions were analyzed using a 6% denaturing polyacrylamide gels.

RESULTS

Dystrophin Protein Studies

Dystrophin analysis was done on the muscle biopsies of the young girl (IV-2), and her male cousin (IV-1). Immunohistochemical staining for dystrophin showed a very mildly reduced signal in a few fibers in the girl, and in numerous fibers in the boy. Dystrophin immunoblotting studies of IV-1 using antibodies directed to the mid-region of dystrophin showed a slightly smaller dystrophin protein of normal quantities (data not shown).

Dystrophin Gene Studies

Multiplex PCR analysis of the dystrophin gene for deletions (18 exons) [Chamberlain et al., 1988; Beggs et al., 1990] showed a deletion of exon 48 in the 4 males with elevations of CK. Quantitative multiplex fluorescent PCR analysis [Schwartz et al., 1992] of exon 48 dosage in all available females showed I-1, II-7, III-1, III-2, III-3, III-5, and IV-2 to be carriers of the exon 48 deletion mutation. To ensure that the failure to amplify exon 48 was not due to a mutation of one of the primer sites in an intron, additional intra-exonic PCR primers were designed for exon 48, and the family re-tested. This analysis verified a deletion of exon 48 (Figs. 1, 2).

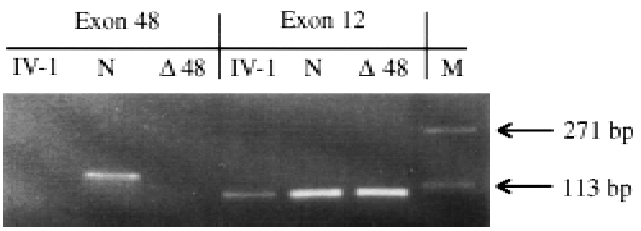

Fig. 2. DNA analysis using intra-exonic primers shows a deletion of exon 48 in the asymptomatic males. Shown are PCR products obtained from intra-exonic PCR primers directed against exon 48 and exon 12. The three individuals shown are an asymptomatic patient from the family (IV-1), a normal control (N), and a Duchenne dystrophy patient with a known deletion of exon 48 (△48).

Linkage analysis was done in the family using multiplex fluorescent CA-repeat analysis with 4 dinucleotide repeat loci distributed throughout the dystrophin gene (Fig. 3). This analysis showed the at-risk haplotype to be "aaaa" in all 7 females carrying the deletion mutation (Fig. 1). Three of the four males with the deletion showed the "aaaa" haplotype; however, a single male (IV-1) showed an "acaa" haplotype. The inheritance of the STR-45 "c" allele suggested either that a meiotic double recombination event occurred in the egg of III-2 giving rise to IV-1 or that there was a new mutation of the STR45 "a" allele which changed it to a "c" allele (Fig. 1). To try to distinguish between these hypotheses additional polymorphic loci were tested in the region of the STR-45 locus (Fig. 3).

STR-44 was not informative. SSCP analysis of the STR-45 locus did not detect any additional sequence changes. Restriction endonuclease digestion of the genomic region pERT-87 with Xmnl was informative but showed inheritance consistent with the "aaaa" maternal hyplotype (not recombinant) (data not shown). These data did not distinguish conclusively between a double recombination and new mutation. If this event was a double recombination, than the two events occurred between exon 17 (pERT-87 Xmnl) and STR45, and between STR45 and exon 48 (deletion) (Fig. 2).

To determine the precise sequence of the mature transcript of the mutant allele, RNA was isolated from muscle biopsies from patient IV-1 and a normal control. RNA was reverse transcribed into cDNA using oligo dT, and the entire coding region of the dystrophin RNA analyzed by RT-PCR (10 fragments using nested PCR; Roberts et al., 1991). Nine of the 10 overlapping regions analyzed showed a single band of the expected size which was identical between the patient and control. The single region containing exon 48 (7a–7b) showed a smaller band in the patient than seen in the control (1120 bp versus 1311 bp)(data not shown). This result was consistent with a deletion of exon 48 in the patient's RNA. Direct sequence analysis of the 7a–7b region from both patient and control showed the expected exon 47/48 junction in the control, while the patient showed sequence indicating an abnormal exon 47/49 junction (Fig. 4). This result demonstrates the deletion of exon 48 and correct in-frame splicing of exon 47 to exon 49.

DISCUSSION

In our patient (IV-2), the mild, persistent elevation of AST, with ALT at the upper limit of normal, pointed to the need to exclude muscle involvement, in spite of the absence of clinical signs and the negative family history. The subsequent finding of an elevated CK confirmed the need for further studies, which were first pursued by electrophysiologic and immunohistochemical study of dystrophin. The results of these tests were diagnostic of a dystrophinopathy: the proposita was found to be a carrier of an exon 48 deletion; four males with high serum creatine kinase levels showed this same deletion mutation. Dystrophin quantity and qual-

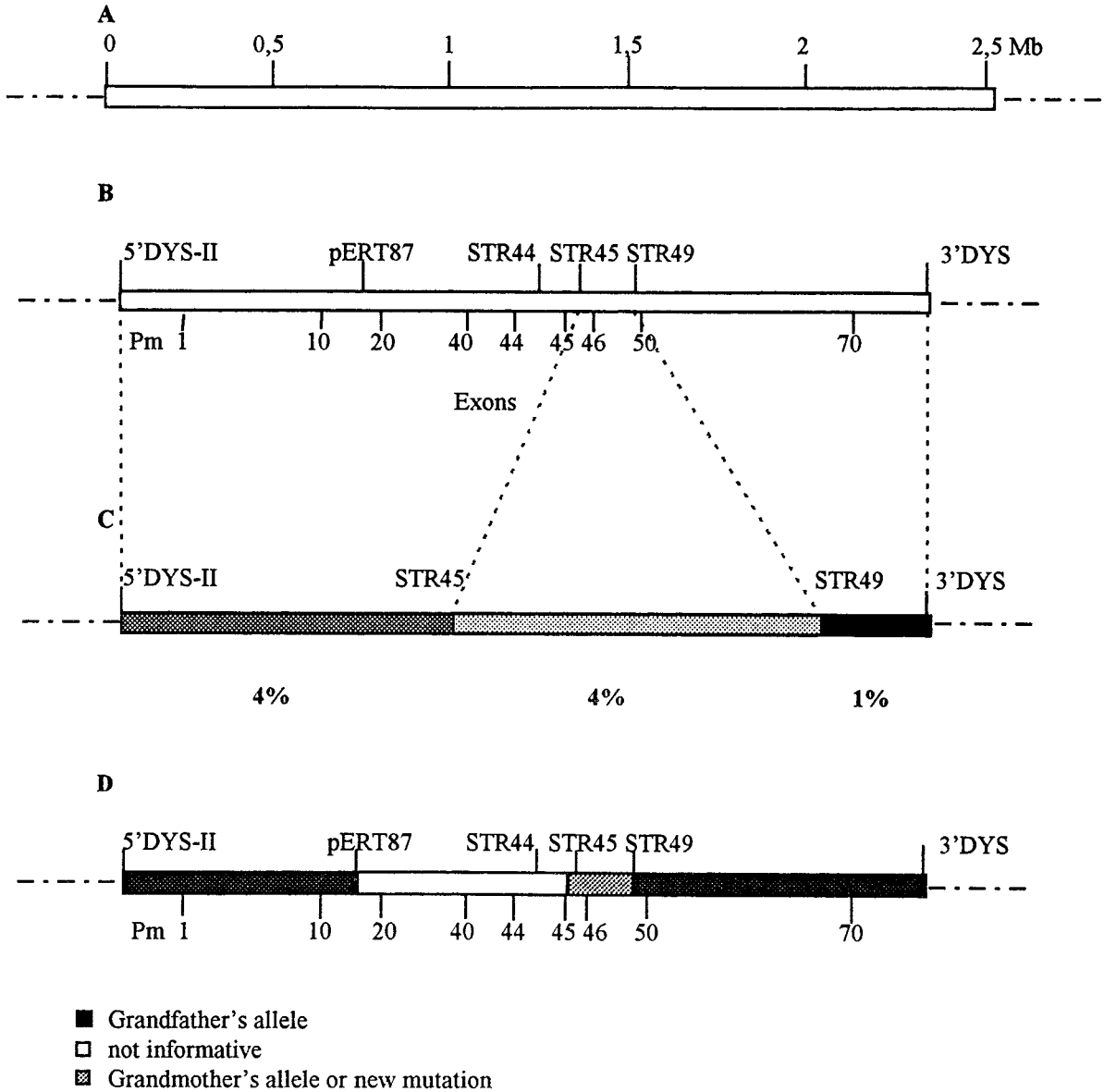

Fig. 3. Dystrophin gene physical and genetic map showing location of polymorphic markers tested. Shown in **A** is a megabase scale map of the dystrophin gene. **B** shows location of polymorphic markers and the physical map. **C** shows the approximate rate of recombination between the dinucleotide repeat markers used in this study. Maps are drawn using data from Oudet et al., 1992. **D** shows a summary of the linkage results for the polymorphic markers. A new mutation or a double recombination event occurred between exon 17 (pERT-87 Xmnl) and STR45, and between STR45 and exon 48 (deletion) in patient IV-1.

ity was abnormal, consistent with the in-frame exon 48 deletion. Finally, the elevated CK and AST levels cosegregated with a specific dystrophin gene haplotype and with the deletion mutation, with the expected X-linked recessive pattern.

The detection of this dystrophinopathy family via AST elevations in a young girl is an unusual aspect of the evaluation of this family. There are two additional aspects which we find remarkable. Recently, an isolated 56-yr-old man with hyperCKemia but no overt clinical symptoms was described with a deletion of exons 50–53 [Comi et al., 1994]. Our study family represents the first description of an extended family segregating an asymptomatic dystrophinopathy in males, some at an advanced age. Other families have been reported in a published abstract [Servidei et al., 1993]. Second, we detected a possible intragenic double recombination event in the dystrophin gene. Unfortunately, testing of many additional loci in the dystrophin gene failed to detect a second locus which also showed recombination, thus we cannot exclude the possibility of a new mutation of the STR-45 "a" allele to a "c" allele which was shared by the other maternal haplotype. Calculation of relative probabilities shows the chance of a double recombination between the exon 48 deletion (proximal boundary) and STR45 (distal boundary) to be about 0.1%, and the chance of a new mutation from the "a" allele of STR45 to a "c" allele to be only a bit less than this.

The identification and characterization of dystrophin

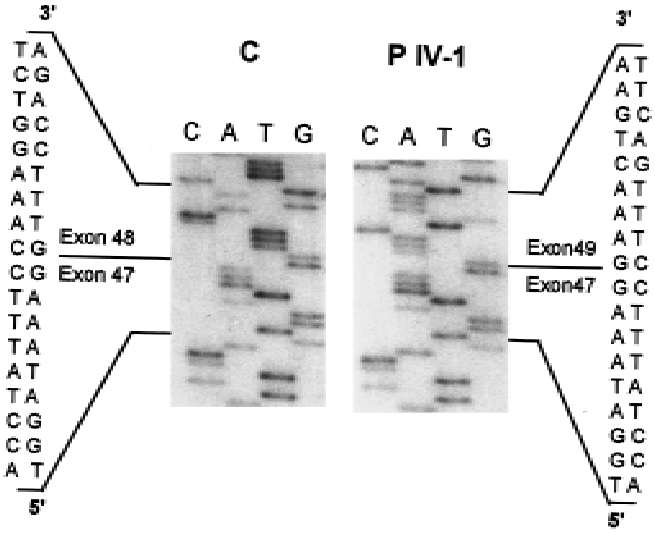

Fig. 4. Direct sequencing of RT-PCR products from patient biopsy shows an in-frame splice junction of exon 47/49. Shown is sequence data from a normal control (c) and a male patient from the pedigree (P IV-1). IV-1 shows a single RNA species with an in-frame deletion of exon 48.

has resulted in new molecular diagnostic classifications of muscular dystrophy, with dystrophin-deficiency (<3% normal levels) considered diagnostic of Duchenne muscular dystrophy, and abnormal dystrophin (abnormal quantity, molecular weight, or both) diagnostic of Becker muscular dystrophy. The clinical range of Becker muscular dystrophy is now recognized to be quite broad, including patients with localized weakness, myalgia and cramps, myoglobinuria, and cardiomyopathy. Despite this range of symptoms, all patients show elevations of serum creatine kinase. To date, a diagnosis of Becker dystrophy implies that muscle weakness will develop at some point in the patient's lifetime.

The males having the exon 48 gene deletion in our study family fulfill molecular criteria for Becker dystrophy, yet were asymptomatic even at relatively advanced ages. Each has an in-frame deletion of the dystrophin gene, and produces dystrophin which is reduced in molecular weight and amount. Other subclinical findings of the patients were consistent with Becker dystrophy: the patients showed a late-onset, subclinical cardiomyopathy, calf hypertrophy, and elevations of serum creatine kinase. However, the patients showed no muscle weakness, despite advanced age (up to 58 yrs). A single patient reported symptoms of myalgia, but only after molecular analysis had established a primary dystrophinopathy. In our experience with 476 Duchenne/Becker patients and families, we have observed this same deletion mutation in two other patients. One was a 5-year-old boy referred for Duchenne muscular dystrophy; he showed progressive proximal muscle weakness, high CK (7000 U/L) and calf hypertrophy. Molecular studies showed a deletion mutation of exon 48. Dystrophin protein analysis was not done. A second patient was a 46-year-old man with a maternal family history of Becker muscular dystrophy. He had muscle hypertrophy, mildly elevated CK (350 U/L, 600 U/L), and complained of myalgias. Dystrophin protein analysis was consistent with Becker muscular dystrophy (390 kDa, 30% normal levels), and DNA analysis showed a deletion of exon 48. Clearly, clinical manifestations of patients having exon 48 deletions are variable. The reasons underlying the clinical variability of this mutation are not known; however, this may be due to alterations in splicing efficiency based upon different deletion breakpoints in the introns, or due to polygenic or environmental factors.

This case highlights some of the ethical dilemmas associated with presymptomatic genetic testing. There is a growing use of molecular methods to diagnose patients before the onset of clinical symptoms. Some presymptomatic testing is done when there is a positive family history, and the prognosis of the presymptomatic patient can often be based on the observed symptoms of older affected relatives. Other presymptomatic testing is done because of incidental laboratory findings (as in the case presented here), or through population-based screening programs. In these latter instances, it is possible to identify mutations in specific genes which infer a "clinical diagnosis" despite the lack of clinical symptoms. In the family presented here, the in-frame deletion of exon 48 of the dystrophin gene, coupled with the elevated serum creatine kinase levels, implies the diagnosis of "Becker muscular dystrophy". Implicit in the diagnosis of Becker muscular dystrophy is "progressive muscle weakness"; however, the affected males in this family were clearly asymptomatic through older ages. This family underscores the danger of labeling presymptomatic patients with a molecular diagnosis. Deletion-positive males in this family could be denied medical insurance coverage under current health care practices in the United States due to the diagnosis of "Becker muscular dystrophy", when in fact they are likely to remain asymptomatic.

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